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Title

Method for examining colorectal cancer and colorectal adenoma

Field of the Invention

5 <0001>

The present invention relates to a method for examining human colorectal cancers and colorectal adenomas, and to antibodies and examination reagents thereof.

Prior Art

10 <0002>

Since the number of colorectal cancer patients is increasing year by year, a method for early detection is necessary. Although, immunological fecal occult blood test and various tumor markers are used at present for the examination of colorectal cancers, these methods do not have satisfactory positive rates. Namely, the positive rate of immunological fecal occult blood test used for the examination of colorectal cancers is 50-60%. As to the tumor markers of colorectal cancers, carcino-embryonic antigen (CEA), CA19-9, STX, which are used for examining the therapeutic effect and for monitoring recurrence, are not satisfactory as tumor markers for early detection of colorectal cancers.

20 <0003>

The colon is divided into right-half and left-half drawing a line at the flexure coli and colorectal cancers in right-half result frequently in pseudo-positive by fecal occult blood test using an anti-hemoglobin antibody, which is pervasively used for the detection of colorectal cancers. Therefore, development of a test method contributing to increased early diagnosis rate particularly of the right-half colorectal cancers is expecting. As to the laboratory diagnosis of colorectal adenomas, which is regarded as the birthplace of colorectal cancers, suitable methods of detecting are not available and people rely inevitably on endoscopy at the present time. Simple method of diagnosing colorectal adenomas will be beneficial to classify patients into groups necessary and unnecessary for endoscopy, and contribute to early detection of colorectal cancers.

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<0004>

Although the sulfation of sugar residues is active in a normal large bowel, it is known to be remarkably reduced in colorectal cancers. Namely, both 3'-sulfation of galactose and 6-sulfation of N-acetylglucosamine (hereinafter referred to as [GlcNAc]), which are abundant in colorectum, are reduced (reference 1). A number of GlcNAc-6-sulfotransferase isozymes have been known in colorectal cancer tissues and in non-cancer colorectal tissues of patients, and I-GlcNAc6ST is significantly decreased in course of carcinogenesis, which leads to the reduced sulfation of sugar residues in colorectal cancer (reference 2). While, GlcNAc6ST-1, one of the isozymes in a normal colorectum, does not show significant changes in the level in course of carcinogenesis. Furthermore, HEC-GlcNAc6ST, another isozyme, increases significantly in colorectal cancer (reference 3).

<0005>

HEC-GlcNAc6ST, which increases in colorectal cancers, synthesizes 6-sulfated GlcNAc and carries out sulfation of GlcNAc in various sugar residues. Therefore, there are a huge variety of the structures of intra-cellularly synthesized sugar residues and their antigenicity. Since GlcNAc6ST-1 and I-GlcNAc6ST also synthesize 6-sulfatedGlcNAc, only the fact that 6-sulfatedGlcNAc is synthesized from HEC-GlcNAc6ST cannot be used as a specific method for diagnosis of colorectal cancer. However, it is known that the substrate selectivity of GlcNAc6ST-1 and I-GlcNAc6ST is more specific than that of HEC-GlcNAc6ST (reference 3, 4). Therefore, certain 6-sulfated sugar residues might be produced by HEC-GlcNAc6ST, but not by GlcNAc6ST nor by I-GlcNAc6ST. However, an actual system for diagnosis of colorectal cancers has not been established.

<0006>

On the other hand, the monoclonal antibody (MECA-79 antibody, reference 5), commercially available as an antibody against an immunological homing receptor of lymphocytes, is known to react with chemically synthesized GlcNAc6-sulfated sugar residues (reference 7). Moreover, the antigens recognizable by the antibody (MECA-79) are reported to emerge on the cell surface, when a mouse gene encoding HEC-GlcNAc6ST enzyme is transduced into CHO cells (hamster ovary cells) (reference 7). However, it has not been known whether the antigens are recognizable by MECA-79 antibody emerged on human cancer cells.

<0007>

reference 1: Izawa, M. et al., Cancer Res., 60: 1410-1416, 2000.

reference 2: Abstract of the 22nd Research Meeting of Japan Molecular Tumor Marker
pp42-43, 2002.

5 reference 3: Seko, A. et al., Glycobiology, 10:919-929, 2000

reference 4: Seko, A. et al., Glycobiology, 12:379-388, 2002

reference 5: Streeter, P.R. et al., J. Cell Biol. 107: 1853-1862, 1988.

reference 6: Bruehl, R.E. et al., J. Biol. Chem. 275: 32642-32648, 2000

reference 7: Yeh, J.C. et al., Cell 105: 957-969, 2001.

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Problems to be solved by the Invention

<0009>

The present invention provides a method for examining colorectal cancer and
colorectal adenoma, which enables to detect colorectal cancer patients and patients
15 at high risk of colorectal cancer at a high probability and is useful for diagnosis of
colorectal cancer and adenoma, and provides the examination reagents thereof.

Means to solve the Problems

<0010>

20 The present inventors discovered that there are significant differences in the
distribution of GlcNAc-6-sulfotransferase isozymes, sulfation enzymes of sugar
residues, between non-cancer colorectal tissues and colorectal cancer tissues or
colorectal adenoma tissues, as a result of investigations. Then the inventors found
that colorectal cancers and adenomas could be detected specifically by assaying
25 6-sulfated sugar residues, which are synthesized only by HEC-GlcNAc6ST, but not
by GlcNAc6ST-1 nor by I-GlcNAc6ST, in tissues of patients and in fecal samples.

Previously, many antibodies such as AG223 (Biochem. (Tokyo), 124:670-678,1998),
G152, G72, AG97, AG107, AG273, G2706, G27011, G27039 (all above,
J.Biol.Chem.,273:11225-11233,1998) are known as those reacting with
30 GlcNAc-6-sulfated sugar residues. Meanwhile, MECA-79 antibody (Pharmingen,
catalog No. 09961D, distributor: Becton Dickinson), which is commercially available
as immunological homing receptor of lymphocytes, is known to react in some way

with GlcNAc-6-sulfated sugar residues (reference 6).

The inventors made a search for an antibody, which have little reactivity to such cells as normal colorectal epithelial cells expressing GlcNAc-6-sulfated sugar residues, but have strong reactivity to cells with such GlcNAc-6-sulfated sugar residues as expressed in cancer cells, by screenings these antibodies. Then the obtained antibodies were assayed against samples from patients and were shown to be highly positive to colorectal cancer cells. The above results lead to completion of the present invention.

<0011>

In other words, the present invention is a method for examining colorectal cancer and colorectal adenoma comprising assaying the reactivity of an antibody to tissues, body fluid or feces of patients, or extracts thereof, wherein said antibodies react with such antigen that is present in cells expressing HEC-GlcNAc6ST gene encoding GlcNAc-6-sulfotransferase and that is absent or almost absent in cells expressing GlcNAc6ST-1 or I-GlcNAc6ST gene. The antigen may be present in cells transduced with HEC-GlcNAc6ST gene and is absent or almost absent in cells transduced with GlcNAc6ST-1 gene or I-GlcNAc6ST gene.

<0012>

The antigen may be represented by the formula below:

R1-Gal β 1-3/4(SO₃-6) GlcNAc β 1-R2

where, R1 represents sugar residues added by other enzymes and is not limited in structure. Gal β represents β galactose, GlcNAc β represents β N-acetylglucosamine, Gal β 1-3/4 represents binding of 1 position of Gal β and 3 position and/or 4 position of GlcNAc β , (SO₃-6) represents addition of a sulfate group to 6 position of GlcNAc β , R2 represents -3GalNAc α , -3Gal β or -2Mana and binds to 1 position of GlcNAc β . As the antibody, MECA-79 antibody (Pharmingen, catalog No. 09961D, shown in Figures 6 and 7) is preferably used.

Also, the present invention is a method for examining colorectal cancer and colorectal adenoma, which comprises reactivity of MECA-79 antibody or its equivalent with tissues, body fluid, feces or extracts thereof of examination subjects.

<0013>

Furthermore, the present invention is any of said methods comprising the reaction of

a labeled probe to said antibody and qualitative or quantitative assay of the label. The preferable method for examination comprises fixing the antigens present in tissues, body fluid or feces or extracts thereof of patients to a membrane, reacting with the antibody, reacting with a labeled probe and detecting the label. It is preferable to insert washing procedures appropriately between the above processes. Said probe includes anti-human-IgG, antibody, protein G, protein A, and protein L. These probes are usually labeled. Said labels include a radioactive isotope (^{125}I) and enzymes (peroxidase, alkaline phosphatase). An antibody with enzyme may involve observation of a change (i.e. color change) by the reaction between the enzyme and the substrate.

<0014>

Moreover, the present invention is an antibody reacting specifically with an antigen carrying sugar residues, which are present in cells expressing HEC-GlcNAc6ST gene encoding GlcNAc-6-sulfotransferase and are absent or almost absent in cells expressing GlcNAc6ST-1 or GlcNAc6ST gene (excluding MECA-79 antibody).

Still furthermore, the present invention is an antibody reacting specifically with an antigen carrying sugar residues, which are present in cells transduced with HEC-GlcNAc6ST gene encoding GlcNAc-6-sulfotransferase and are absent or almost absent in cells transduced with GlcNAc6ST-1 or GlcNAc6ST gene (excluding MECA-79 antibody).

<0015>

Still moreover, the present invention is an antibody (excluding MECA-79 antibody) reacting specifically with an antigen carrying sugar residues, which are present in tissues, body fluid or feces of patients with colorectal cancers and colorectal adenomas and expressed by the following general formula:

$\text{R1-Gal } \beta 1-3/4 (\text{SO}_3-6) \text{ GlcNAc } \beta 1-\text{R2}$

(in the formula, each note is the same to the above).

Also, the present invention is an examination reagent of colorectal cancer and colorectal adenoma comprising any of these antibodies, including MECA-79 antibody, as a main component.

Brief Description of the Drawings

<0008>

Figure 1 shows the results of flowcytometric analysis using MECA-79 antibody on colorectal cancer cells (COLO201 cells) and on normal colorectal epithelial cells (SW480 cells treated with Tricostatine A). a shows the enzyme specificity of colorectal cancer cells (COLO201 cells) and normal colorectal epithelial cells (SW480 cells). b shows the results of flowcytometric analysis on the reactivity of anti-6-sulfated sugar residues antibody with the above 2 kinds of cells. The ordinate shows the cell frequency (the number of cells) and the abscissa axis shows the fluorescence (Arbitrary Unit).

Figure 2 shows the result of the flowcytometric analysis on the reactivity of MECA-79 antibody with cells transduced with HEC-GlcNAc6ST gene, GlcNAc6ST-1 gene or I-GlcNAc6ST gene. The ordinate shows the cell frequency (the number of cells) and the abscissa axis shows the fluorescence (Arbitrary Unit).

Figure 3 shows the photographs of tissues stained with MECA-79 antibody of a colorectal cancer patient. Ca shows a colorectal cancer tissue and N shows non-cancer colorectum tissue.

Figure 4 shows the photographs of tissues of colorectal cancer and normal colorectum of a patient stained with MECA-79 antibody. Ca shows cancer tissues and N shows non-cancer colorectum tissues in a to c. N shows a non-adenomatous colorectum tissue and A shows adenomatous cells in d. The black part (brown in color) shows the presence of antigens recognizable by the antibody and the gray part (faint blue in color) shows control staining with methylene blue.

Figure 5 shows the reactivity of MECA-79 antibody with fecal extracts of patients. Each row from above shows colorectal cancer 8 cases, colorectal adenoma 8 cases, benign 8 cases and healthy control 8 cases, respectively.

Figure 6 shows a catalog of MECA-79 antibody (Pharmingen, catalog No. 09961D).

Figure 7 shows a catalog of MECA-79 antibody (Pharmingen, catalog No. 09961D).

Detailed Description of the Invention

5 <0016>

The structure of 6-sulfated sugar residues, which are little synthesized by GlcNAc6ST-1 or by I-GlcNAc6ST, but are synthesized only by HEC-GlcNAc6ST is expressed as the following general formula:

R1-Gal β 1-3/4 (SO₃-6) GlcNAc β 1-R2.

10 <0017>

GlcNAc β , which is the substrate of GlcNAc6-sulfotransferase in a body, is carried by various sugar residue carrier. R2 shows the carrier.

HEC-GlcNAc6ST has been known to transfer sulfate residues to all kinds of GlcNAc β 1-R2 previously tested according to both our research and other people's research (references 4 and 7). In contrast, GlcNAc6ST-1 and I-GlcNAc6ST transfer sulfate residues only to such GlcNAc β -R2 as accompanied with a specific form of R2. The case, which HEC-GlcNAc6ST but not GlcNAc6ST-1 nor I-GlcNAc6ST can transfer sulfate residues, is known as a case when R2 is -3GalNAc α (the structure after sulfation is SO₃-6GlcNAc β 1-3GalNAc α), a case when R2 is -3Gal β (the structure after sulfation is SO₃-6GlcNAc β 1-3Gal β) and a case when R2 is -2Man α (the structure after sulfation is SO₃-6GlcNAc β 1-2Man α) (J. Biol. Chem., 277: 3979-3984, 2002 and Glycobiology, 12: 379-388, 2002). In the examination method of the present invention, a specific antibodies to any of the three cases and antibodies cross reacting to all three sugar residues may be usable.

25 <0018>

GlcNAc-6-sulfotransferase adds sulfate group to distal GlcNAc of sugar residues and synthesizes 6-sulfated GlcNAc (i.e. SO₃-6GlcNAc) intra-cellularly. However, after synthesis of distal 6-sulfated GlcNAc of sugar residues, the modified sugar residues are further added sugar residues (R1) by other enzyme groups intra-cellularly, and a large variety of the structure and antigenicity of the sugar residues are finally synthesized and produced from cells. Generally the structure added to 6-sulfated GlcNAc is Gal β 1-4 and Gal β 1-3 (referred to as Gal β 1-3/4). Moreover, it is known that

NeuAc α 2-3/6, SO₃⁻ 3/6, and Fuc α 1-2/3/4 are added to the 6-sulfated GlcNAc. The R1 part is added after the synthesis of 6-sulfated GlcNAc by GlcNAc-6-sulfotransferase. Therefore, R1 part is not related to the substrate specificity of such GlcNAc-6-sulfotransferases as HEC-GlcNAc6ST, GlcNAc6ST-1 and I-GlcNAc6ST.

5 <0019>

The antigens with the above sugar residues are present in cancer tissues obtained from colorectal cancer patients by biopsy or by surgical operation, and present in such samples as serum, ascites and feces containing the antigens. Also, the antigen may be easily extracted from these samples using phosphate buffered saline. Also, 10 the antibody against this sugar residue antigen could be obtained by known arts producing antibodies (e.g. Methods in Enzymology, 312: 160-179, 2000; Methods in Molecular Biology, 199: 203-218, 2002 et al.).

<0020>

The GlcNAc-6-sulfated sugar residues detected by the present invention are positive 15 not only in colorectal cancers but also in colorectal adenomas, which are regarded as the birthplace of colorectal cancers. Therefore, using the sugar residues as a target of screening test, a group of patients with colorectal adenomas, for whom endoscopic examination or follow-up is required, could be detected. Compared with the occult blood test with anti-hemoglobin antibody, which is currently used for screening of 20 colorectal cancers, the present method has higher yield of detection of colorectal adenomas. Also, 6-sulfated sugar residues are originally abundant in right-half of colorectum, if a colorectum is separated in right-half and left-half, therefore the above method of diagnosis is particularly useful for the diagnosis of colorectal cancers and colorectal adenomas generated in right-half colorectum. Since colorectal 25 cancers in right-half are not frequently positive by fecal occult blood test using anti-hemoglobin antibody, concomitant use of the present method may contribute to increased yield of positive diagnosis in right-half colorectal cancers.

The following examples are provided to illustrate the present invention, but are not 30 intended to limit the scope thereof.

Example 1

<0021>

Gene expression of GlcNAc-6-sulfotransferase isozymes was examined by RT-PCR on human-derived colorectal cancer cells and on normal colorectal epithelial cells.

- 5 In the RT-PCR analysis, PCR primers for detection of the expression of HEC-GlcNAc6ST gene (Genebank, AF131235) are synthetic oligonucleotides of SEQ ID NO. 1 for upper strand side and those of SEQ ID NO. 2 for lower strand side ($T_m=59^{\circ}\text{C}$), those for GlcNAc6ST-1 gene (Genebank, AB011451) are synthetic oligonucleotides of SEQ ID NO. 3 for upper strand side and those of SEQ ID NO. 4 for
10 lower strand side ($T_m=62^{\circ}\text{C}$) and those for I-GlcNAc6ST gene (Genebank, AF176838) are synthetic oligonucleotides of SEQ ID NO. 5 for upper strand side and those of SEQ ID NO. 6 for lower strand side ($T_m=60^{\circ}\text{C}$).

The results are shown in Figure 1a. COLO201 cells are typical cells showing colorectal cancer pattern, which expresses strongly HEC-GlcNAc6ST gene and little
15 GlcNAc6ST-1 and I-GlcNAc6ST genes. TSA-SW480 cells treated SW480 cells with tricostatinA are typical cells showing normal epithelial pattern, which little expresses HEC-GlcNAc6ST gene, but significantly expresses GlcNAc6ST and I-GlcNAc6ST genes.

<0022>

- 20 Then, a number of anti6-sulfated sugar residues antibody were screened based on the reactivity to the above two kinds of cells. In other words, antibodies, which react well with COLO201 cells and not react with TSA-SW480 cells, were searched. The screening of the reactivity between cells and antibodies was performed by flowcytometric analysis by use of FACScan (Becton Dickinson) stained cells with
25 indirect fluorescent antibody method (the first antibody 1.0 $\mu\text{g}/\text{ml}$, 4°C , 30 min, the second antibody rabbit anti-rat IgM antibody (Zymed Laboratories), 4°C , 30 min).

A typical result of the analysis is shown in Figure 1b. MECA-79 antibody (Pharmingen, catalog No. 09961D, Distributor: Becton Dickinson) showed strong reactivity with COLO201 cells, but showed little reactivity with TSA-SW480 cells.
30 The above result showed that MECA-79 antibody is a preferable antibody for diagnosis of colorectal cancers. While, G72 antibody used as a control (J. Biol. Chem., 273: 11225-11233, 1998) reacted significantly with both COLO201 cells and

TSA-SW480 cells and is not appropriate for diagnosis of colorectal cancers.

Example 2

<0023>

5 In this example, cells transduced with HEC-GlcNAc6ST gene, GlcNAc6ST-1 gene or I-GlcNAc6ST gene were prepared. Then flowcytometric analysis for these cells was performed using MECA-79 antibody.

For the preparation of cells transduced with HEC-GlcNAc6ST gene, the gene (Genebank, AF131235) inserted into pCDNA3.1 vector was used. For those with
10 GlcNAc6ST-1 gene, the gene (Genebank, AB011451) inserted into pIRES1hygro vector was used. For those with I-GlcNAc6ST gene, the gene (Genebank, AF176838) inserted into pCDNA3.1 vector was used. The flowcytometric analysis was performed as in Example 1.

The results are shown in Figure 2. MECA-79 antibody reacted strongly with those
15 cells transduced with HEC-GlcNAc6ST, but reacted with slightly those cells transduced with GlcNAc6ST gene or I-GlcNAc6ST gene.

Example 3

<0024>

20 Colorectal cancer tissues derived from patients (31 cases) were stained with immunohistological staining using MECA-79 antibody. For immunohistological staining, frozen sections with 10 µm thick were used, 1.0 µg/ml MECA-79 antibody was used as the first antibody, and a reagent kit (Vectastain) of Vecta Co. containing anti-rat IgM antibody was used as the second antibody according to the protocol of
25 the company.

The results are shown in Figure 3. The antibody does not react with non-cancer colorectal mucosa (N) (0 case/31 ca31 cases, 32 %). Positive reaction rate is higher for cancers in right-half colorectum (60%) and lower for those in left-half colon (19 %).

<0025>

30 A typical stained photographs are shown in Figure 4.

Figure 4a shows the stained photograph of a colorectal cancer tissue (ca) and a colorectal non-cancer tissue (N) of a patient. The colorectal cancer tissue is strongly

stained but the non-cancer tissue is little stained.

Figure 4b shows the photograph of the same tissue stained using AG107 antibody. Since AG107 antibody reacts with general GlcNAc-6-sulfated sugar residues, the non-cancer tissue (N) was stained well much more than the cancer tissue (Ca) in contrast to 4a and GlcNAc-6-sulfated sugar residues only could not be used for the specific detection of cancer tissues. Namely, use of such antibody as MECA-79, which detects the specific GlcNAc-6-sulfated sugar residues abundant in colorectal cancers and colorectal adenomas, could be applied for the specific detection.

Figure 4c shows the example of the expression of the sugar residues in a colorectal cancer tissue derived from right-half colorectum. That the cancer tissue is strongly stained shows strong expression of the sugar residues.

Figure 4d shows the expression in an adenomatous polyposis coli. N shows non-adenomatous colorectal tissue and A shows adenomatous cells. The adenomatous part of A is well stained by MECA-79 antibody. GlcNAc-6-sulfated sugar residues, which could be detected by MECA-79 antibody, are expressed abundantly in non-cancer adenomatous polyposis as well as in cancer tissues. Adenomatous polyposis coli, which is benign by itself but is regarded as the birthplace of colorectal cancer, could be detected credibly.

Example 4

<0026>

In this example, an enzyme-linked immunosorbent assay, which is a simple qualitative test and uses MECA-79 antibody for the reaction with fecal extracts of colorectal cancer patient, confirmed the emergence of GlcNAc-6-sulfated sugar residues in feces of patients. Sometimes sugar residue antigens are decomposed by enzymes secreted from fecal bacterium and could not be detected in feces. Therefore, the confirmation is important for the practicability of the present invention.

0.1 g human feces was dispersed in 1 ml fecal extraction buffer (10 mM PBS, 1 % BSA, pH 7.5), centrifuged at 8,000g for 15 min at 4°C, and the supernatant was recovered.

To 40 µl supernatant, 120 µl extraction buffer was added and the sample was prepared. The sample was blotted to a PVDF membrane (Immobilon, Milipor, Lot K2JN2659B) by suction and the membrane was reacted with MECA-79 antibody,

rabbit anti-rat IgM antibody, POD labeled goat anti-rabbit IgG antibody, and avidin-biotin complex solution, sequentially, after blocked for nonspecific reactions, and stained in NTB solution.

The results are shown in Figure 5.

- 5 Positive results are 4 cases in 8 cases of colorectal cancers (50 %) and 4 cases in 8 cases of colorectal adenomas (50 %). For benign disorders cases, 1 case gives a slight positive result and almost all cases of normal healthy subjects are negative.